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PRINCIPAL INVESTIGATOR: Parkash Gill, M.D.

CONTRACTING ORGANIZATION: University of Southern California
Los Angeles, CA 90089

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14. ABSTRACT Multiple receptor pathways are often induced in cancer and thus allow redundancy in providing growth and survival advantage to tumor cells. In addition, micro-environment including increased blood perfusion into the tumor can provide advantage to the tumor. EphB4 receptor tyrosine kinase & its ligand EphrinB2 appear to have dual function. EphB4 & its ligand are needed for the formation & maturation of blood vessels. EphB4 in addition is induced in tumor cells, where it may cooperate with HER2 growth factor signaling. Thus co-targeting HER2 & EphB4 could lead to significant therapeutic benefits. The goal of this project is to assess the in vitro and in vivo growth and signaling effects of co-targeting using approved anti-HER2 agents, trastuzumab & lapatinib in combination with an inhibitor of EphB4-EphrinB2, namely soluble EphB4-HSA, which was discovered in our laboratory. The other component of this project is to use genetic models to define the contribution of EphB4 & EphrinB2 in the formation and progression of Her2 positive tumor. In this aim we will use Her2 transgenic mice that develop mammary gland tumors, and cross with the mice in which either EphB4 or EphrinB2 gene is deleted. We have generated or acquired the mouse lines to conduct this part of the project. Lastly we wish to determine if EphB4 is induced in tumors that become resistant to Her2 targeted therapy, and the role of EphB4 in such a case. Can EphB4 targeted therapy reset the clock and sensitize the tumor to her2 targeted therapy. Early evidence indicates that this may be possible. This work & the soon to start Phase I study of soluble EphB4HSA phase I trial at our institution will set the foundation for design of combined targeting Her2 & EphB4 in human trial.					
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INTRODUCTION:

We propose that the EphB4 receptor tyrosine kinase and its cognate ligand EphrinB2, while normally regulate the development and maturation of newly forming vessels are aberrantly expressed in Her2 positive breast cancer. Under these circumstances EphB4 and EphrinB2 co-operate with Her2 positive breast cancer and promote HER2 signaling. Thus co-targeting HER2 and EphB4 could lead to significant therapeutic benefits.

The aims of the project are to determine in vitro and in vivo contribution of EphB4-EphrinB2 in the tumor development and progression in the context of Her2 over-expression. We anticipate that EphB4-EphrinB2 will enhance signaling downstream from Her2 pathway, and reversal of the signaling events when co-targeting Her2 with agents such as trastuzumab or lapatinib and EphB4-ephrinB2 inhibitor (sEphB4-HSA). sEphB4-HAS is a novel agent developed by our group, a ligand-blocking soluble albumin-stabilized EphB4 peptide termed sEphB4-HSA. In this line of investigation we also wish to determine if EphB4-EphrinB2 targeting may sensitize the tumor to otherwise resistance to Her2 targeted therapy.

The other component covered by this project by the Partnering PI is to investigate the human tissue in patients who received pre-operative therapy with or without the HER2-targeted antibody trastuzumab. Markers of angiogenesis, including EphB4 and its cognate ligand EphrinB2 and downstream signaling will be analyzed for the relationship to response to Her2 targeted therapy and to determine if enrichment of these markers occurs in non-responders from the initial biopsy to the post-treatment surgical specimen. The demonstration of these human tissue effects along with the efficacy of in vitro and in vivo co-targeting of HER2 and EphB4 will set the stage for clinical trial strategies as sEphB4-HSA is already in Phase I testing at our institution.

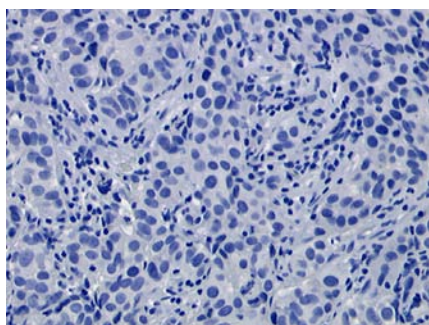
BODY:

Task 1. Partnering PI Dr. Tripathy has obtained tissue blocks on the 42 HER2+ cases, including baseline core biopsies and surgical specimens from patients who did not exhibit a pCR. A total of 64 blocks has been retrieved. For paired pre/post samples in non-pCR cases (needed for Task 3E), of the 24 non-pCR cases, 14 paired breast samples are available. 20 additional HER2+ cases that received neo-adjuvant therapy and had their diagnostic biopsy, treatment and surgery at our institution have been identified and the tissue retrieval is needed.

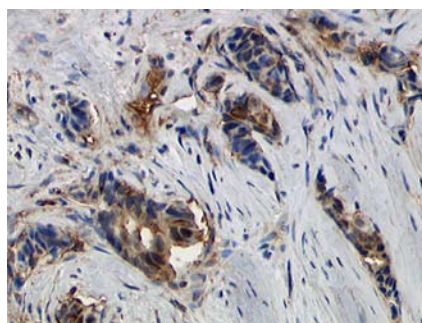
Task 2A. Staining for H&E for the presence of tumor cells has been done on all 64 blocks retrieved. The planned immunohistochemical (IHC) stains are EphB4, EphB2, HGFR/c-MET, IGF-1R, PDGFR, VEGFR1 and VEGFR2. We have decided to add Ki67 and CXCR4. We are ahead of schedule on this task, having all the staining, scanning and scoring done on EphB4 and

EphrinB2, all the staining and scanning done for c-MET, IGF1-R and staining for PDGFR. Representative stains for EphB4 is shown below.

Representative IHC Stains for EphB4 in a Pretreatment Case



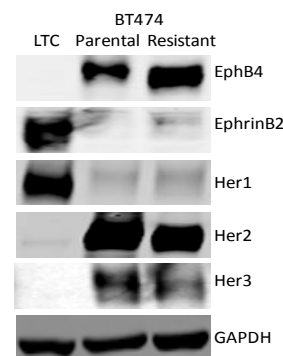
EphB4negative – 20X



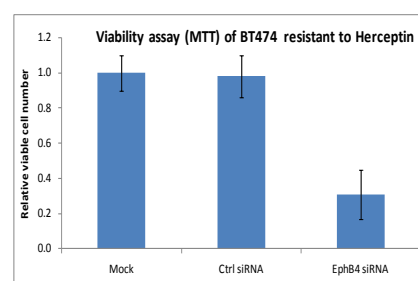
EphB4, positive – 20X

Task 2C. We produced anti-human EphB4 and EphrinB2 antibodies in CHO cells. Antibodies were purified on Protein A column and characterized for purity and potency. We provide antibodies to VEGF and VEGF receptors. Other antibodies were obtained from various vendors. IHC protocols for EphB4, EphB2, EphrinB2, VEGF, VEGF receptors have been developed and optimized in our laboratory in the past. These protocols were optimized for IHC protocols for use in human tissues. All other antibodies were obtained, methodology including processing, antibody titer and optimization of antibodies was established as described in the grant proposal.

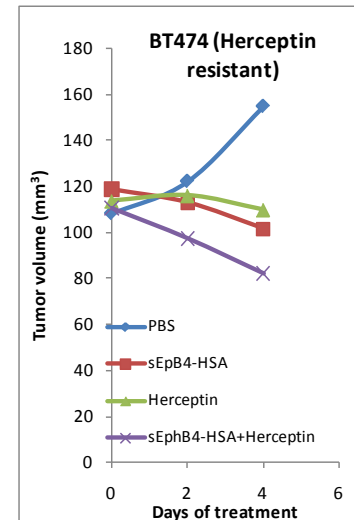
Her2 positive tumor cell lines resistant to Hereceptin: In order to determine the role of EphB4 in resistant breast cancer, we are developing Herceptin resistant Her2 positive cell lines in collaboration with Dr. Tripathy. Compared to Her2 positive BT474 primary cell, Herceptin resistant cell line shows significant increase in the levels of EphB4, suggesting a potential role of EphB4 in Herceptin resistance (see figure on the right).



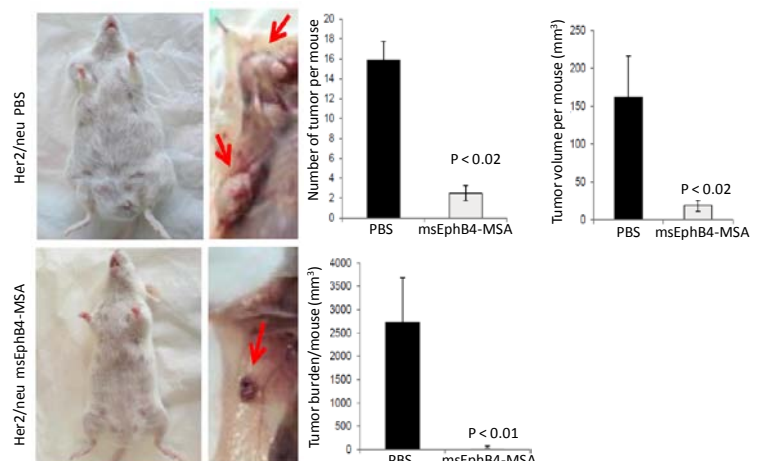
In order to determine if EphB4 is providing a survival signal to BT474 cell line, we knocked down EphB4 using siRNA. EphB4 siRNA inhibits EphB4 expression by over 80% compared to control siRNA. Cell viability studies show that EphB4 siRNA, markedly reduced the cell viability of Her2 positive Herceptin resistant BT474 cell, in sharp contrast to control siRNA (see figure on the right).



EphB4 targeted therapy in Her2 positive Herceptin resistant tumor in vivo: We next wished to determine if Her2 positive Herceptin resistant tumor responds to EphB4 targeted therapy, and sensitize tumor to Herceptin. In order to test these two possibilities, we are conducting in vivo studies with Herceptin resistant Her2 positive BT474 cell. 5 million Herceptin resistant BT474 cells were implanted in athymic Balb/C mice. Tumors were allowed to establish. Mice were randomized to four groups to receive PBS (control), sEphB4-HSA, Herceptin or combination of sEphB4-HSA and Herceptin. This ongoing experiment will provide evidence for potential efficacy of sEphB4-HSA in Herceptin resistant tumors. If the combination therapy of sEphB4-HSA and Herceptin is superior to sEphB4-HSA and Herceptin alone, an evidence for reversal of Herceptin resistance is likely. Tissue analysis for total and phosphorylated Her2, EGFR, EGFR3, EphB4, Akt, S6, MAPK will be informative. Furthermore analysis for cell proliferation, cell death, blood vessel density will help determine the mechanism of action of sEphB4-HSA.



Task 3. We have conducted in vivo studies to determine the efficacy of inhibitor of EphB4-EphrinB2 using soluble EphB4 receptor extra-cellular domain. Thus coding region corresponding to amino acid 1 to 527 at the N-terminus and full length human serum albumin coding region at the C-terminus (sEphB4-HSA) were expressed in CHO cells and protein was purified to homogeneity. We have previously used human sEphB4 fused in frame with full length human serum albumin coding region for in vivo studies. In studies of safety of sEphB4-HSA in mice, we have determined that immune competent mice generate antibodies to human sEphB4-HSA. Due to the need for long term use of the therapeutic compound, we produced murine EphB4-murine serum Albumin (msEphB4-MSA). Purified msEphB4mSA was used on mutant Her2 transgenic mice from the age of 20-25 weeks during which time mice develop tumors. Six mice per group were treated and the tumor volumes from drug vs control PBS treatment were measured. Significant tumor inhibition was observed in msEphB4mSA treatment group (see figure below). In addition mice were treated with lapatinib that blocks kinase activity of EGFR and Her2. As expected Lapatinib inhibited tumor growth in mutant Her2 transgenic mice (data not shown).



We have obtained the antibodies for the analysis of the drug and control

PBS treated mice tumor to study activated HER2/HER family receptors, angiogenic markers (EphrinB2, EphB1,2,3,4,6, VEGF, VEGFR-1, 2, 3 and PDGFR), vessel density (CD31), signal transduction pathways (Akt, mTOR, S6, MAPK), proliferation (Ki-67) and apoptosis (TUNEL) and optimized the immunohistochemistry protocol. These studies will be conducted during the next several months.

The assessment of the effects of sEphB4-HSA on the same markers along with cell viability and DNA incorporation in breast cancer cell lines and mammosphere preps will be conducted during the next 6 months.

Task 4. We have obtained or generated EphrinB2 and EphB4 flox/flox allele in mouse for targeted deletion in the mammary gland. This will be accomplished by crossing these mice with the transgenic mouse lines expressing Cre, and mutant Her2 in the mammary gland. These studies will define the role of EphB4 and EphrinB2 in the Her2 directed mammary tumor. We have initiated these studies, but the mutant her2 transgenic mice from the control group did not generate tumors. We have to start this work with another breeding pair of mice to first ensure that the control group develops tumors. We will then cross with other transgenic lines. Thus Task 4 has resulted in unexpected delays.

Task 5. We have joint meetings once a month to outline work plan, conduct the studies, and review the data. We will continue this process during the next year. sEphB4-HSA is moving towards the clinic first to define the safety, dose, schedule and potential. Ongoing work and analysis will guide us to the clinical strategy and the development of the treatment protocol. Results from Phase I study of sEphB4-HSA will allow us to refine the clinical development plan in Her2 positive breast cancer especially those who fail Her2 targeted therapy.

KEY RESEARCH ACCOMPLISHMENTS:

- Production of antibodies to EphB4 and EphrinB2 in our laboratory. Acquisition of other reagent antibodies. Development and optimization of IHC protocol. Analysis of tissues for EphB4 and EphrinB2.
- Development of Her2 positive Her2 antibody resistant tumor cell line. Demonstration of induction of EphB4 and contribution of EphB4 in tumor resistance. In vivo studies to test efficacy of EphB4-EphrinB2 targeted therapy and sensitization to Her2 targeted therapy in progress.
- Development of mouse cell line for conditional deletion of EphB4 and acquisition of EphrinB2 cell line for conditional deletion. Acquisition of mouse cell line expressing cre-enzyme in mammary tissue in order to conditionally delete EphB4 or EphrinB2 in mammary gland. Acquisition of mutant her2 transgenic line that develops mammary tumors spontaneously, however another breeding pair will be obtained to ensure tumor development in controls prior to breeding with other mouse transgenic lines.

- Design and production of murine version of soluble EphB4 fused to mouse serum albumin (murine sEphB4-MSA) for in vivo studies in immune competent mice. In vivo efficacy studies of murine sEphB4-MSA in mutant her2 transgenic mouse.

REPORTABLE OUTCOMES:

We have successfully applied for a proposal that is related to this strategy to co-target EphB4 and the Notch receptor, using an antibody discovered by Dr. Gill laboratory to the tumor-specific Notch ligands DLL-1 and DLL-4. (California Breast Cancer Research Program Project entitled “Co-Targeting the Notch and EphB4 Receptor in Breast Cancer” (CBCRP 18IB-0048)). This project received the highest scientific score of all review submissions.

CONCLUSION:

We have already begun to show that EphB4 is expressed in Her2 positive tumor tissues. We plan to determine if outcome to Her2 targeted therapy has a correlation with EphB4 or EphrinB2 expression. In addition correlation with other angiogenic factors is planned. We have also determined in vitro that tumor cell line resistant to Her2 targeted antibody has induction of EphB4 and that EphB4 provides survival advantage based on the results of EphB4 knock down in this cell line BT474. Additional Her2 positive cell lines resistant to Her2 antibody will be generated and studied as well. In vivo studies of Her2 positive Her2 antibody resistant tumor cell line for response to EphB4 targeted therapy with sEphB4-HSA for direct effect and possible sensitization to Her2 directed therapy is in progress. Additional cell lines resistant to Her2 targeted therapy will similarly be investigated. Studies using genetic models of spontaneous Her2 positive tumor in transgenic mouse when crossed with EphB4 or EphrinB2 knock out mouse will provide orthogonal evidence for the role of EphB4 in Her2 positive breast cancer. This work will be done in the next several months. These studies will establish the basis for clinical trials co-targeting EphB4 and HER2. EphB4-targeted therapy with sEphB4-HSA developed in our laboratory is entering a Phase I trial at our institution during the next few weeks.